Co-treatment with panobinostat and JAK2 inhibitor TG101209 attenuates JAK2V617F levels and signaling and exerts synergistic cytotoxic effects against human myeloproliferative neoplasm cells

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ABSTRACT

The mutant JAK2V617F tyrosine kinase (TK) is present in the majority of patients with BCR-ABL negative myeloproliferative neoplasms (MPNs). JAK2V617F activates downstream signaling through the STAT, RAS/MAPK and PI3/AKT pathways, conferring proliferative and survival advantages in the MPN hematopoietic progenitor cells (HPCs). Treatment with the pan-histone deacetylase (HDAC) inhibitor panobinostat (PS) is known to inhibit the chaperone function of heat shock protein 90, as well as induce growth arrest and apoptosis of transformed HPC’s. Here, we demonstrate that PS treatment depletes the auto-phosphorylation, expression and downstream signaling of JAK2V617F. Treatment with PS also disrupted the chaperone association of JAK2V617F with hsp90, promoting proteasomal degradation of JAK2V617F. PS also induced apoptosis of the cultured JAK2V617F-expressing human erythroleukemia HEL92.1.7 and Ba/F3-JAK2V617F cells. Treatment with the JAK2 TK inhibitor TG101209 attenuated JAK2V617F autophosphorylation and induced apoptosis of HEL92.1.7 and Ba/F3-JAK2V617F cells. Co-treatment with PS and TG101209 further depleted JAK/STAT signaling and synergistically induced apoptosis of HEL92.1.7 and Ba/F3-JAK2V617F cells. Co-treatment with TG101209 and PS exerted greater cytotoxicity against primary CD34+ MPN cells than normal CD34+ HPCs. These in vitro findings suggest combination therapy with HDAC and JAK2V617F inhibitors is of potential value for the treatment of JAK2V617F positive MPN.
INTRODUCTION

Philadelphia-chromosome negative myeloproliferative neoplasms (MPNs) are a group of clonal hematopoietic disorders that includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1,2). Recent studies have confirmed the pathogenetic involvement of an acquired, somatic, gain-of-function, activating, point mutation JAK2V617F in MPNs (3-6). This represents a guanine to thymidine mutation in exon 14 resulting in a valine to phenylalanine substitution at codon 617 in the JH2 or pseudokinase domain of the JAK2 gene (a member of the Janus kinase (JAK) family of non-receptor tyrosine kinases, JAK1, JAK2, JAK3 and TYK2) (2,6). Highly sensitive assays for JAK2 have determined that the JAK2V617F mutation is present in 90% of patients with PV and approximately 50-60% of patients with ET or PMF (7). In addition, a subset of patients, most commonly with PV, are homozygous for the JAK2V617F allele, the result of copy-neutral loss of heterozygosity at the JAK2 locus, especially in patients with PV (2,7,8). Mutations in exon 12 of JAK2 are present in almost all patients with PV who are JAK2V617F negative (9,10). The JAK proteins function in the cytoplasm to relay signals initiated by membrane bound cytokine receptors. Engagement of the receptor results in the phosphorylation of the receptor and JAK2, which recruits its substrate proteins such as signal transducers and activators of transcription (STATs) (11,12). STATs, especially STAT3 and STAT5, translocate to the nucleus and transactivate many genes involved in cell proliferation and survival, e.g., Bcl-xL, cyclin D1 and PIM1 (8,11,12). The V617F mutation in JAK2 also activates the downstream signaling pathways through the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK). This contributes to diminished apoptosis of the HPCs (2,8). Over-expression of JAK2V617F in murine Ba/F3 cells with co-expression of the erythropoietin receptor (EpoR) confers in vitro cytokine-independent growth (3,13). Recently, it
was shown that enforced expression of JAK2V617F in human HSCs and myeloid progenitors directed differentiation toward the erythroid lineage, along with increased expression and phosphorylation of GATA-1 and decreased expression of PU.1 (14-16). JAK2V617F expression in retroviral models and in transgenic mice is sufficient to cause myeloproliferative disorders in the mice that recapitulate many clinicopathologic features observed in human PV, ET and PMF (17-21). Therefore, the mutant JAK2 represents an excellent target for therapeutic intervention in MPNs. Several, orally bio-available, small molecule, ATP-competitive, JAK2-selective inhibitors have been tested in pre-clinical studies and are undergoing clinical testing in MPNs (22). Pre-clinical studies have shown that treatment with JAK2-selective kinase inhibitors, e.g., TG101209 (TG) and TG101348, attenuate p-JAK2 levels, as well as inhibit JAK2 induced p-STAT5, p-STAT3, p-AKT and p-ERK1/2 levels in cultured and primary human MPN cells with JAK2V617F mutation (22,23). In vivo studies in mouse models have also shown that mutant JAK2V617F represents a novel target for therapeutic intervention with JAK2-selective tyrosine kinase inhibitors in MPNs (21,24). For example, TG101348 inhibits myeloproliferation and myelofibrosis in a murine model of JAK2V617F-induced polycythemia (21,22). Early clinical trials of several JAK2-selective kinase inhibitors, e.g., XL019, TG101348 and INCB18424, are underway in JAK2-driven MPNs with poor prognosis, e.g., PMF (22,25). Preliminary results suggest that selective JAK2 inhibitors are relatively well-tolerated, ameliorate constitutional symptoms including pruritis and fatigue, reduce splenomegaly, but have so far not shown the ability to reverse myelofibrosis or to eradicate the JAK2V617F mutant clone (2,23,25). Short of allogeneic stem cell transplantation, curative therapies that confer a survival benefit are not available, thereby creating a need for better therapies for MF-MPN (26).
Panobinostat (LBH589, PS) is a cinnamic acid hydroxamate capable of inhibiting class I and class II histone deacetylases (HDACs), thereby inducing the acetylation of both histone and non-histone proteins (27-31). Treatment with HDAC inhibitors (HDIs), e.g., PS, has been shown to induce cell cycle inhibition, growth arrest and apoptosis of human leukemia cells, which is correlated with increased expression of p21, p27 and pro-death Bcl-2 family of proteins, as well as concomitant attenuation of p-AKT, c-RAF and anti-apoptotic Bcl-2 family of proteins (27-32). Importantly, PS exerts a relatively sparing effect on normal bone marrow progenitor cells (28,29). PS-mediated hyperacetylation and inhibition of the chaperone function of hsp90 directs its client proteins, e.g., BCR-ABL, RAF-1 and AKT, to polyubiquitylation and proteasomal degradation (27-31).

Recently, ITF2357 (a class I and II HDI) was shown to selectively down modulate the levels of JAK2V617F protein and its downstream signaling through p-STAT3 and p-STAT5 in human MPN cells (33). Importantly, ITF2357 also reduced splenomegaly and constitutional symptoms, and induces hematologic responses in some patients with PV/ET (34). In the present studies, we determined the effects of PS alone and in combination with TG in mouse and human bone marrow cells containing the mutant JAK2V617F. Our findings demonstrate that PS treatment inhibits the expression levels, activity and downstream pro-growth and pro-survival signaling of JAK2V617F. Additionally our findings show that combined treatment with PS and TG induces synergistic apoptosis of HEL92.1.7 (HEL) cells and exhibits superior activity against primary MF-MPN cells.

**Methods and Materials**

**Reagents and antibodies:** Panobinostat (PS) was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). TG101209 (TG) was kindly provided by TargeGen Inc. (San Diego, CA). Cycloheximide was obtained from Sigma Aldrich (St. Louis, MO). Anti pJAK2 (Tyr1007/1008),
anti-JAK2, anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727), anti-STAT3 anti-pAKT (Ser473), anti-AKT and polyclonal GATA-1 were obtained from Cell Signaling (Beverly, MA). Monoclonal anti-pSTAT5 (Tyr694), monoclonal anti-c-RAF, and Bcl-xL, were obtained from BD Transduction Labs (San Jose, CA). Polyclonal anti-STAT5 was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal anti-pGATA-1(Ser310) was obtained from Novus Biologicals (Littleton, CO). Rat monoclonal anti-hsp90 antibody was obtained from StressGen Biotechnologies (Vancouver, British Columbia). Polyclonal anti-pERK1/2 and anti-ERK1/2 were obtained from Invitrogen Life Sciences (Carlsbad, CA). Acetylated K69 hsp90 antibody was generated as previously described (31). Monoclonal anti-β-actin was obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and cell culture. HEL 92.1.7 (HEL) and murine Ba/F3-EpoR-JAK2V617F, and Ba/F3-EpoR cells were maintained in RPMI media with 10% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids (11,23). Ba/F3-hEpoR cells were supplemented with 10% WEHI preconditioned media. Logarithmically growing cells were exposed to the designated concentrations of TG101209 and/or panobinostat. Following these treatments, cells or cell pellets were washed free of the drug(s) prior to the performance of the studies.

Primary MF-MPN cells: Primary peripheral blood and/or bone marrow aspirate MF-MPN samples were obtained with informed consent according to the Declaration of Helsinki from patients with high risk MF (≥ 3, according to the International Prognostic Scoring System, IPSS) (1,35). This was sanctioned by a clinical protocol approved by the Institutional Review Board of the Medical College of Georgia. The samples were collected in heparinized tubes, and mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (Stem Cell Technologies, Vancouver, British Columbia), washed once and re-suspended in complete RPMI-
1640, and counted to determine the number of cells isolated prior to their use in the experiments (28). Banked, de-linked and de-identified, donor peripheral blood CD34+ mononuclear cells procured from recipients who had since deceased and primary MF-MPN cells were purified by immuno-magnetic beads conjugated with anti-CD34 antibody prior to utilization in the cell viability assay (StemCell Technologies). Human Primitive Hematopoietic Progenitor Cell Enrichment Kit (StemCell Technologies) was used to obtain stem cells. Briefly, the mononuclear fraction from MF-MPN was incubated with StemSep Enrichment Cocktail (containing monoclonal antibodies to lineage markers such as CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD56, CD66b, glycophorin A and conjugated to anti-dextran antibodies) and incubated for 15 minutes at room temperature. Following this, the mixture was incubated with magnetic colloid, mixed and further incubated for 15 minutes at room temperature. The mixture was loaded onto a primed column and placed inside a magnet to eliminate all cells that expressed markers in the Enrichment Cocktail. CD34+CD38-Lin− cells were obtained from the column flow through during column washing with 2% FBS containing PBS. CD34+CD38− stem cells were immunophenotyped by staining with CD34-PE and CD38-FITC then analyzed by flow cytometry for the percentage enrichment and purity using a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA).

**RNA isolation and quantitative polymerase chain reaction.** Untreated or drug treated HEL cells were centrifuged to pellet the cells. Total RNA was extracted using an RNAqueous RNA kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Two micrograms of RNA were reverse transcribed using a first strand synthesis kit from Invitrogen (Carlsbad, CA). The resulting cDNAs were mixed with 10μL of 2X TaqMan Universal Master mix (Applied Biosystems, Foster City, CA) and TaqMan probes (containing primers and 5 pmol of 5-carboxyfluorescein (FAM)
fluorescently labeled probe) for the exon 8-9 and exon 23-24 boundary of JAK2 (Applied Biosystems, Foster City, CA). All samples and loading controls were plated in triplicate, centrifuged briefly, and then loaded onto a StepOne 9600 Real Time PCR system (Applied Biosystems, Foster City, CA). Amplifications were performed using the following PCR cycling conditions. Samples were heated to 95°C for 10 minutes, then 40 cycles of denaturation at 92°C (15 seconds) and annealing and extension step at 60°C (1 minute). Amplified products were normalized against GAPDH expression.

**Assessment of apoptosis by annexin-V staining.** Untreated or drug-treated cells were stained with Annexin-V (Pharmingen, San Diego, CA) and propidium iodide (PI) and the percentage of apoptotic cells were determined by flow cytometry. To analyze synergism between TG101209 (TG) and panobinostat in inducing apoptosis, cells were treated with TG (200-800 nM) and panobinostat (5-20 nM) at a constant ratio of 40:1 for 48 hours. The percentage of apoptotic cells was determined by flow cytometry, as previously described (27,29). The combination index (CI) for each drug combination was calculated by median dose effect analyses (36), utilizing the combination index equation within the commercially available software CalcuSyn (Biosoft, Ferguson, MO). CI values of less than 1.0 represent synergism of the two drugs in the combination.

**Assessment of percentage non-viable cells.** Following designated treatments, cells were stained with trypan blue (Sigma, St. Louis, MO). The numbers of non-viable cells were determined by counting the cells that showed trypan blue uptake in a hemocytometer, and reported as percentage of untreated control cells.

**Determination of JAK2 protein half life.** HEL cells (5 x 10^6) were left untreated or treated with 5 μg/mL of cycloheximide (CHX), 50 nM of PS, or the combination of PS and cycloheximide for
0, 4, 8, 16, and 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2 and β-actin. Representative immunoblots were used for densitometric analysis to assess the percent of JAK2 remaining in each treatment group.

**Cell lysis and protein quantitation.** Untreated or drug-treated cells were centrifuged, and the cell lysates were obtained from cell pellets and incubated on ice for 30 minutes, as previously described (28). After centrifugation, an aliquot of each cell lysate was diluted 1:10 and protein quantitated using a BCA protein quantitation kit (Pierce, Rockford, IL), according to the manufacturer’s protocol.

**Immunoprecipitation of hsp90 and JAK2 and immunoblot analyses:** Following the designated treatments, immunoprecipitation and immunoblotting of hsp90 was performed as previously described (30). For the immunoprecipitation of JAK2 from total cell lysates, 500 μg of total cell lysate was used with 0.5 μg of rabbit monoclonal anti-JAK2 antibody (Cell Signaling, Beverly, MA). Protein A-agarose beads were used to pull down the immunoprecipitates. The beads were washed four times in lysis buffer, and then boiled in SDS sample buffer prior to SDS-PAGE and immunoblot analyses.

**SDS-PAGE and Western Blotting:** One hundred micrograms of total cell lysate was used for SDS-PAGE. Western blot analyses of pJAK2 (Tyr1007/1008), JAK2, pSTAT3 (Tyr705), pSTAT3 (Ser727), STAT3, pSTAT5 (Tyr 694), STAT5, pAKT (Ser473), AKT, Bcl-xL, pGATA(Ser310), GATA, pERK1/2 and ERK1/2 were performed on total cell lysates using specific antisera or monoclonal antibodies, as previously described (27-31). The expression level of β-actin was used as the loading control for the Western blots. Blots were developed with a chemiluminescent substrate ECL (Amersham Biosciences, Piscataway, NJ).
Statistical Analysis: Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined using the Student’s t-test. P values of less than 0.05 were assigned significance.

Results

Panobinostat (PS) inhibits JAK2V617F expression and signaling and induces apoptosis of mouse and human HPCs expressing JAK2V617F. We first determined the effects of clinically achievable concentrations of PS on the viability of the cultured human erythroleukemia HEL cells and the mouse pro-B Ba/F3-hEpoR and Ba/F3-hEpoR-JAK2V617F cells with or without the ectopic expression of JAK2V617F (23). As demonstrated in Figure 1A, treatment with panobinostat (10-30 nM) induced apoptosis of HEL and Ba/F3-JAK2V617F cells in a dose dependent manner. Conversely, panobinostat exerted significantly less cytotoxic effects against Ba/F3-hEpoR cells without the expression of JAK2V617F (Figure 1A). We next determined the effects of PS on the expression and signaling downstream of JAK2V617F in HEL cells. Treatment with PS inhibited the autophosphorylation and levels of JAK2V617F in a dose dependent manner with near-complete loss of JAK2 phosphorylation at Tyr1007/1008, following exposure to 30 nM of PS (Figure 1B). Inhibition of JAK2 activity by PS was associated with inhibition of p-STAT3 and p-STAT5, with greater reduction in STAT3 than STAT5 levels (Figure 1B). Treatment with PS also reduced the levels of p-GATA1, p-AKT and p-ERK1/2, with concomitant decline in the levels of GATA1 and AKT but not ERK1/2 (Figure 1B). Inhibition of STAT-5 signaling was associated with decline in the levels of Bcl-xL, as has also been previously reported (Figure 1B) (26). Caspase-3 activity leading to apoptosis, as induced by PS, is also known to cause processing of Bcl-xL, which likely contributed to the decline in the levels of Bcl-xL (37). Treatment with PS
induced similar effects on p-JAK2, as well as on levels of p-STAT3 and p-STAT5 in Ba/F3-JAK2V617F cells (Figure 1C and data not shown). In contrast, PS treatment did not result in loss of p-JAK2 or p-STAT5 and p-STAT3 in Ba/F3-hEpoR cells (Supplemental Figure 1A). We next determined whether PS treatment also inhibited the mRNA expression of JAK2. As shown in Figure 1D, treatment with even low level of PS (5 nM) resulted in approximately 40% depletion of the mRNA expression of JAK2 as determined by the real-time polymerase chain reaction with two independent primer/probe sets located at different exon boundaries within the mRNA. Exposure to higher concentrations of panobinostat did not further reduce the expression of JAK2 mRNA (Figure 1D). We have previously demonstrated that PS treatment induces hyperacetylation and inhibition of the chaperone function of hsp90 resulting in proteasomal degradation of hsp90 client proteins (e.g., BCR-ABL, AKT and RAF1) (30,31). Results of our present studies show that immunoprecipitates of hsp90 and JAK2 showed binding of JAK2 to hsp90 and was partially disrupted by exposure to PS (Figure 2A). Consistent with this, PS treatment promoted proteasomal degradation and partial depletion of JAK2 and RAF1, which was reversed by co-treatment with the proteasome inhibitor bortezomib (Figure 2B). These findings indicate that PS-mediated depletion of JAK2 levels is partly due to inhibition of mRNA and partly from increased JAK2 protein degradation. We next determined the half life of the JAK2 protein in HEL cells. Treatment with cycloheximide caused a time-dependent decline in the JAK2 protein levels with a 50% reduction in expression by 18 hours (Figure 2C). Co-treatment with PS and cycloheximide resulted in a more rapid decline in JAK2 expression levels (Figure 2C). The findings demonstrate that treatment with PS shortened the half life of JAK2 by approximately 60%, from 18 to 7 hours (Figure 2C).

TG inhibits the activity and downstream signaling of JAK2V617F and induces apoptosis of mouse and human HPCs expressing JAK2V617F. We next determined the effects of TG in
cultured bone marrow progenitor cells expressing JAK2V617F. Treatment with TG (0.2-2 µM) dose-dependently induced apoptosis of HEL cells (Figure 3A). TG also induced significantly more apoptosis of Ba/F3-JAK2V617F versus Ba/F3-hEpoR cells (Figure 3A). We next determined the effect of TG on JAK2 expression and signaling. Unlike panobinostat, treatment with TG did not significantly alter the mRNA expression of JAK2 in HEL cells (Figure 3B). Although treatment with TG inhibited p-JAK2, p-STAT3 and p-STAT5, significant attenuation of p-JAK2, JAK2, STAT3 and STAT5 levels was observed in HEL cells only following exposure to 2.0 µM of TG. Treatment with TG also reduced Bcl-xL, p-AKT and p-GATA1 levels in HEL cells, which correlated with TG-induced apoptosis of HEL cells (Figure 3C). Similar to the observations in HEL cells, TG also inhibited the downstream signaling due to JAK2V617F in the Ba/F3 cells (Figures 3D and 4B). Treatment with TG markedly depleted p-JAK2, p-STAT3, p-STAT5 and Bcl-xL levels, without significantly depleting JAK2V617F, STAT3 and STAT5 levels. Additionally, TG treatment attenuated p-AKT and p-GATA1 levels in Ba/F3-JAK2V617F cells. We also determined the effects of TG in Ba/F3-hEpoR cells. Treatment with TG had minimal effects on p-JAK2, p-STAT5 and p-AKT levels in the Ba/F3-hEpoR cells. However, TG treatment significantly inhibited p-STAT3 in the Ba/F3-hEpoR cells (Supplemental Figure 1B).

**Co-treatment with TG and PS causes greater inhibition of JAK/STAT activity and induces synergistic apoptosis of mouse and human HPCs expressing JAK2V617F.** We next determined the effects of co-treatment with TG and PS in Ba/F3-JAK2V617F and HEL cells. Figure 4A demonstrates that co-treatment with TG (200 or 1000 nM) and PS (10 nM) induced more apoptosis of Ba/F3-JAK2V617F cells than either agent alone. Both TG and/or PS induced
more apoptosis of Ba/F3-JAK2V617F versus Ba/F3-hEpoR cells (Figure 4A). Immunoblot analyses following treatment with TG or TG plus PS (20 nM) demonstrated that co-treatment with TG and PS caused greater depletion of p-STAT5 and p-AKT than TG alone in Ba/F3-JAK2V617F cells (Figure 4B). A similar effect was also observed against p-STAT3 (data not shown). Combined treatment with TG and PS also decreased the levels of STAT5 and AKT, especially at higher dose levels of TG (Figure 4B). In contrast, co-treatment with TG and PS exerted similar but less pronounced effects than TG alone on p-STAT5, p-AKT and p-STAT3 (not shown) in Ba/F3-hEpoR cells (Figure 4C). Co-treatment with 10 nM PS also significantly increased apoptosis of HEL cells induced by 500 or 1000 nM of TG (p < 0.05) (Figure 5A). Importantly, the combined treatment with PS and TG synergistically induced apoptosis of HEL cells, as determined by median dose effect analysis of Chou and Talalay (Figure 5B). Combination index (CI) values for the drug combinations were less than 1.0, indicating a synergistic interaction at concentrations that were below the IC50 values for PS (5-20 nM) and TG (200-800 nM) (Figure 5B). Consistent with these observations, co-treatment with 20 nM of PS markedly increased TG (0.2 or 1.0 µM)-mediated attenuation of p-JAK2, JAK2, p-STAT5, p-STAT3, p-AKT and p-GATA1 levels in HEL cells (Figure 5C), as compared to treatment with TG or PS alone (Figure 1B).

**PS and TG inhibit JAK/STAT signaling and exert greater anti-MF-MPN effects than either agent alone in primary MF-MPN HPCs expressing JAK2V617F.** We next determined the effects of PS and/or TG on the viability of primary CD34+ MF-MPN cells from patients with MF due to JAK2 V617F and normal CD34+ HPCs. Treatment with TG caused a dose-dependent increase in the loss of viability of CD34+ primary MF-MPN cells (Figure 6A). A similar effect
was also observed with PS treatment alone (data not shown), with approximately 50% of the cells determined to be non-viable following treatment with 20 nM of PS (Figure 6A). Co-treatment with 20 nM of PS significantly enhanced TG-induced cell death of CD34+ primary MF-MPN cells. In contrast, treatment with PS alone or co-treatment with PS and TG induced significantly less cell death in normal human CD34+ HPCs, as compared to CD34+ primary MF-MPN cells (Figure 6A). In a representative sample yielding adequate numbers of CD34+ primary MF-MPN cells for immunoblot analysis, treatment with PS dose-dependently depleted JAK2, p-STAT5 and p-STAT3, p-ERK1/2 and p-AKT levels, without significantly affecting STAT5, STAT3, AKT and ERK1/2 levels (Figure 6B). As previously reported for human breast cancer cells, treatment with PS also induced hyper-acetylation of lysine (K) 69 on hsp90 in the primary CD34+ MF-MPN cells (Figure 6B). In the same sample of primary CD34+ MF-MPN cells, co-treatment with TG and PS resulted in greater inhibition of STAT5 and STAT3 phosphorylation than either agent alone, as determined by immunoblot analysis of total cell lysates (Figure 6C). CD34+CD38-Lin- cells from three MF-MPN patients were also treated with the indicated concentrations of TG101209 (TG) and/or panobinostat (PS) for 48 hours (Figure 6D). Following treatment, the percentages of non-viable cells were determined by trypan blue dye uptake in a hemocytometer. As shown in Figure 6D, treatment with 20 nM PS induced more cell death of MF-MPN stem cells than treatment with either 200 or 1000 nM of TG. Additionally, co-treatment with PS and TG also induced significantly more cell death of bone marrow stem cells than either agent alone (p < 0.05). These findings demonstrate that combined treatment with PS and TG would also be effective in exerting lethal action against bone marrow derived stem cells with mutant JAK2V617F expression.
Discussion

In the present studies, we demonstrate that treatment with PS inhibits the autophosphorylation and expression of JAK2V617F, as well as its downstream signaling in the cultured mouse Ba/F3, human erythroleukemia HEL cells, and primary MF-MPN HPCs. While our studies neither identified the transcription factor involved nor elucidated the mechanism by which the transcription of JAK2V617F is affected, it is clear that PS depleted the mRNA levels as well as promoted the proteasomal degradation of JAK2V617F, which together contributed to the overall decline in the levels of JAK2V617F and its downstream signaling. These findings are consistent with the previously reported observation in which PS and other pan-HDAC inhibitors were shown to deplete BCR-ABL and FLT-3 levels in human leukemia cells both by transcriptional and post-transcriptional mechanisms (27,29-31). Because these agents also inhibit HDAC6, pan-HDAC inhibitors such as PS induce hyper-acetylation of hsp90, thereby inhibiting its chaperone function and promoting the polyubiquitylation and proteasomal degradation of hsp90 client proteins, including BCR-ABL, FLT-3, AKT, RAF1 and CDK4 (27,31). The PS mediated down regulation of JAK2 protein is relatively modest, even though PS inhibits transcription of JAK2 and promotes its degradation by the proteasome. This is mainly due to the relatively long half-life (approximately 18 hours) of the protein. Disruption of JAK2V617F binding to hsp90 due to PS treatment and restoration of the levels of JAK2V617F by co-treatment with bortezomib supports the conclusion that JAK2V617F is also an hsp90 client protein. This was also supported by the observations that the geldenamycin analogue hsp90 inhibitor or AUY922 also partially deplete JAK2V617F in the MF-MPN HPCs (data not shown). It is also increasingly being recognized that the mutant oncoprotein kinases are more dependent on the chaperone association
with hsp90 than their unmutated counterparts, for example as noted for BCR-ABL, FLT-3, EGFR and B-RAF (27,29,39,40). Consequently, treatment with pan-HDAC or hsp90 inhibitors has been shown to be more effective in depleting the mutant versus the unmutated forms of these oncoprotein kinases. Our finding that PS treatment depletes JAK2V617F more than unmutated JAK2, as observed in Ba/F3-hEpoR cells, is consistent with these reports. Treatment with PS was also noted to inhibit JAK2V617F-mediated downstream signaling, as highlighted by PS-mediated depletion of the levels of p-STAT5, p-AKT, p-GATA1 and pERK1/2. This may not only be partly due to the direct inhibitory effects of PS on JAK2V617F, but may also be due to the known PS-mediated depletion of p-AKT and p-ERK1/2, or through the effects of PS on other upstream signaling kinases. It should also be noted that although down-regulation of JAK2 protein by PS is relatively modest, the inhibition of phosphorylation of JAK2 and its downstream targets e.g., STAT3 and STAT5, AKT, and ERK1/2 is more prominent. There are two potential reasons for this finding. One could be a technical reason based on the relative difference in the epitope detection by the specific antibodies for the un-phosphorylated versus phosphorylated forms of the protein. The other, more likely, reason could be that panobinostat (PS)-induced misfolding of JAK2 could affect more, and earlier, the detection of the phosphorylated epitope than the lowering of JAK2 by proteasomal degradation, which ensues later and is regulated by other factors. Regardless, the likely net effect of PS was to attenuate the pro-growth and pro-survival signaling more in HEL and Ba/F3-JAK2V617F than in Ba/F3-hEpoR cells. Additionally, because HEL and Ba/F3-JAK2V617F cells are more dependent on this signaling, PS also induced significantly more apoptosis of HEL and Ba/F3-JAK2V617F than of Ba/F3-hEpoR and normal CD34+ human HPCs. A similarly selective effect of the pan-histone deacetylase inhibitor ITF2357 was also reported against JAK2V617F and HEL cells (33).
Importantly, in the present studies the in vitro inhibitory effects of PS on p-JAK2 V617F, p-STAT3 and p-STAT5 were also confirmed in patient-derived CD34+ MF-MPN cells.

Our findings also demonstrate that, as compared to treatment with either agent alone, combined treatment with PS and TG is more effective in attenuating not only the mutant JAK2V617F, p-STAT3 and p-STAT5 but also p-AKT, and p-GATA1 levels, especially when PS was combined with lower levels of TG (200 nM). This was associated with a significant increase in apoptosis, which suggests that down regulation of multiple survival mechanisms contributes to the lethal effects of the combination in cells which have endogenous expression of JAK2V617F. Combined treatment also induces significantly more apoptosis of HEL, Ba/F3-JAK2V617F and primary CD34+ MF-MPN cells than of Ba/F3-hEpoR and CD34+ normal human HPCs with unmutated JAK2. This observation mimics what was also noted when combination of pan-HDAC inhibitors such as PS or vorinostat, or the hsp90 inhibitor 17-AAG, was used in combination with BCR-ABL or FLT-3 TK inhibitor in CML and AML cells that expressed the mutant forms of BCR-ABL or FLT-3 (27,29). Previous reports have described the individual activity of TG101209 (TG) and TG101348 against JAK2V617F-expressing human MPN cells and Ba/F3 cells (21-24). Based on the more pronounced inhibitory effects of the combination of PS and TG on the levels and signaling downstream of JAK2V617F, there is clearly the potential for accruing additional in vivo therapeutic advantages due to treatment with the combination versus treatment with JAK2 TK inhibitors alone. High level of expression and deregulated activity of JAK2V617F in HPCs can stimulate homologous recombination, genomic instability and increased centrosome and ploidy abnormalities (41). High intracellular levels of reactive oxygen species (ROS) have also been noted that may contribute to genomic instability and
disease progression (42,43). In this context, it is noteworthy that compared to treatment with each of the agents alone combined treatment with PS and TG exhibited higher lethal activity against the CD34+, CD38-, Lin- primary MF-MPN stem cells with mutant JAK2V617F. Although not directly investigated here, it is also likely that PS-mediated anti-hsp90 activity and superior activity of the combination of PS and TG would exert greater anti-PIM/BAD/Bcl-xL effect downstream of JAK2V617F, since PIM kinase is also known to be an hsp90 client protein (19,44-46). This would also attenuate the resulting PIM-mediated survival signaling and MYC function in MF-MPN cells (19,45,46). It is also important to note that anti-hsp90 effects of co-treatment with PS and TG may retard the emergence of any other mutant JAK2 clones which could potentially confer resistance against treatment with a JAK2 TK inhibitor alone, as has been observed with BCR-ABL and FLT-3 TK inhibitors in CML and AML cells (47-49).

Early clinical trials with TG101348 and other JAK2 TK inhibitors suggest a promising clinical benefit of these agents in patients with MF-MPN (22,25). However, complete remissions similar to those seen in CML with BCR-ABL TK inhibitors have not as yet been observed. Recently, in a phase I clinical trial of PS in a wide spectrum of hematologic malignancies, clinical benefit was observed in patients with MF-MPN (38,50). Based on this, a phase II multi-institution clinical trial of PS is being implemented in patients with advanced MF-MPN (50). Taken together with the findings presented here these encouraging developments support the rationale to design and implement future clinical studies of PS and JAK2 TK inhibitors in patients with advanced MF-MPN.
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Authorship:


Conflict of Interest Disclosure:

Co-author P.A. is an employee of Novartis Institute for Biomedical Research Inc. and the corresponding author, K.N.B., has received clinical and laboratory research support from Novartis Institute for Biomedical Research Inc. A.P.J. received research support from Novartis. All other authors have no competing financial interests.
References


43. Walz C, Cross NC, Van Etten RA, Reiter A. Comparison of mutated ABL1 and JAK2 as oncogenes and drug targets in myeloproliferative disorders. Leukemia. 2008; 22(7): 1320-34.


Figure Legends

Figure 1. Treatment with panobinostat (PS) inhibits JAK2 mRNA expression, JAK/STAT signaling and induces apoptosis of MPD cells and Ba/F3 cells with ectopic over expression of JAK2V617F. A. HEL, Ba/F3-JAK2V617F, and Ba/F3-hEpoR cells were treated with the indicated concentrations of panobinostat (PS) for 48 hours. Following this, the cells were stained with Annexin V and propidium iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of three independent experiments; Bars represent standard error of the mean. B. HEL cells were treated with the indicated concentrations of PS for 24 hours. Then, total cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), pSTAT3 (Ser727), STAT3, Bcl-xL, pAKT (Ser473), AKT, pERK1/2, ERK1/2, pGATA-1 (Ser310) and GATA-1. The expression levels of β-actin in the lysates served as the loading control. C. Ba/F3-JAK2 V617F cells were treated with the indicated concentrations of PS for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), and STAT3. The expression levels of β-actin in the lysates served as the loading control. D. HEL cells were treated with the indicated concentrations of PS for 16 hours, then mRNA was isolated and reverse transcribed. Quantitative real-time PCR was performed on the cDNA using TaqMan probes for the exon 8-9 boundary and exon 23-24 boundary of JAK2. Relative expression of JAK2 mRNA was normalized to GAPDH expression.

Figure 2. Treatment with PS inhibits JAK2 binding with hsp90 leading to proteasomal degradation of JAK2 in MPD cells. A. HEL cells were treated with the indicated
concentrations of PS for 8 hours. Then, cell lysates were prepared and hsp90 was immunoprecipitated. The immunoprecipitates were separated by SDS-PAGE and immunoblot analyses were performed for JAK2 and hsp90. Alternatively, JAK2 immunoprecipitates were prepared and immunoblot analyses were performed for hsp90 and JAK2. B. HEL cells were treated with the indicated concentrations of PS and/or bortezomib (BZ) for 4 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for JAK2 and c-Raf (RAF1). The expression levels of β-actin in the lysates served as the loading control. C. HEL92.1.7 cells were treated with the indicated concentrations of cycloheximide (CHX) and panobinostat (PS) for 0, 8, 16, and 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2. The expression levels of β-actin in the lysates served as the loading control.

**Figure 3. Treatment with TG inhibits JAK2 phosphorylation and downstream signaling and induces apoptosis in HEL and Ba/F3-JAK2 V617F cells.** A. HEL, Ba/F3-JAK2 V617F, and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG for 48 hours. After treatment, the cells were stained with Annexin V and propidium iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of three independent experiments; Bars represent standard error of the mean. B. HEL cells were treated with the indicated concentration of TG for 16 hours. Total RNA was harvested and reverse transcribed, then used for quantitative real time polymerase chain reaction for JAK2 expression. Relative expression of JAK2 was normalized to GAPDH. C. HEL cells were treated with the indicated concentrations of TG for 24 hours. Then, cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5,
pSTAT3 (Tyr705), STAT3, Bcl-xL, pAKT (Ser473), AKT, pERK1/2, ERK1/2, pGATA-1 (Ser310) and GATA-1. The expression levels of β-actin in the lysates served as the loading control. C. Ba/F3-JAK2V617F cells were treated with the indicated concentrations of TG for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, Bcl-xL, pAKT (Ser473), AKT, pERK1/2, and ERK1/2. The expression levels of β-actin in the lysates served as the loading control.

**Figure 4. Co-treatment with PS enhances TG mediated inhibition of JAK2 downstream signaling and induction of apoptosis in Ba/F3 cells with ectopic overexpression of JAK2 V617F.** A. Ba/F3-JAK2V617F and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG and/or PS for 48 hours. After treatment, the cells were washed with 1X PBS and stained with Annexin V and propidium iodide, and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of three independent experiments; Bars represent standard error of the mean. B-C. Ba/F3-JAK2V617F and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG and/or PS for 24 hours. Following this, cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, pAKT (Ser473) and AKT. The expression levels of β-actin in the lysates served as the loading control. A vertical line has been inserted to indicate a repositioned gel lane.

**Figure 5. Co-treatment with PS enhances TG-mediated inhibition of JAK2 phosphorylation and downstream signaling and induces synergistic apoptosis of MPD cells.**
A. HEL cells were treated with the indicated concentrations of TG and/or PS for 48 hours. Then, the cells were washed with 1X PBS and stained with Annexin V and propidium iodide, and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of three independent experiments; Bars represent standard error of the mean. B. HEL cells were treated with TG (200-800 nmol/L) and PS (5-20 nmol/L) for 48 hours. Apoptosis was determined by Annexin V staining and flow cytometry. Median dose effect and combination indices were obtained using CalcuSyn software. CI values less than 1.0 indicate synergism of the two agents. C. HEL cells were treated with the indicated concentrations of TG and/or PS for 24 hours. Following treatment cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, pGATA-1 (Ser310) and GATA-1 and pAKT (Ser473). The expression levels of β-actin in the lysates served as the loading control.

Figure 6. Co-treatment with PS and/or TG exerts greater down regulation of JAK2 and downstream signaling, as well as greater cytotoxicity than either agent alone in primary MF-MPN cells. A. CD34+ progenitor cells isolated from primary MF-MPN peripheral blood samples and normal CD34+ bone marrow progenitor cells were treated with the indicated concentrations of TG and/or panobinostat for 48 hours. After treatment, the percentages of non-viable cells were determined by trypan blue dye uptake in a hemocytometer. * indicates values significantly greater (p<0.05) than treatment with either agent alone. B. Primary MF-MPN cells were treated with the indicated concentrations of PS for 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, Bcl-xL, pAKT (Ser473), AKT, pERK1/2, ERK1/2, and acetylated K69 hsp90.
The expression levels of β-actin in the lysates served as the loading control. **C.** Primary MF-MPN cells were treated with the indicated concentrations of TG and/or panobinostat for 24 hours. Then, cell lysates were prepared and immunoblot analyses were performed for JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705) and STAT3. The expression levels of β-actin in the lysates served as the loading control. **D.** Primary CD34⁺CD38⁻Lin⁻ stem cells from MF-MPN patients were treated with the indicated concentrations of TG and PS for 48 hours. Following treatment, the percentages of non-viable cells were determined by trypan blue dye uptake in a hemocytometer.
Figure 1

A

% apoptosis

nM, PS, 24 hours

HEL 92.1.7

Ba/F3-JAK2V617F

Ba/F3-hEpoR

B

HEL 92.1.7

nM, PS, 24 hours

p-JAK2(Tyr1007/1008)

JAK2

p-STAT5 (Tyr694)

STAT5

p-STAT3α/β (Tyr705)

p-STAT3α (Ser727)

STAT3

Bcl-xL

p-AKT(Ser473)

AKT

p-ERK1/2

ERK1/2

p-GATA1(Ser310)

GATA1

β-actin

D

mRNA expression (% of control)

nM, PS, 16 hours

JAK2 exon 8-9

JAK2 exon 23-24

1.0      0.76     0.71     0.49

1.0      0.88     0.83     0.62

1.0      0.80     0.78     0.53

1.0      0.90     0.87     0.54

β-actin
Figure 2

A. HEL 92.1.7

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B. HEL 92.1.7

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C. HEL 92.1.7

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<td>CHX + PS 50 nM</td>
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Figure 3

A

![Bar chart showing % apoptosis of HEL 92.1.7, Ba/F3-hJAK2V617F, and Ba/F3-hEpoR cells at different concentrations of TG (μM) after 48 hours.](image)

B

![Bar chart showing %mRNA expression (relative to control) of JAK2 exon 8-9 and JAK2 exon 23-24 at 0.5 μM TG.](image)

C

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D

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Figure 4

A

![Bar graph showing percentage of apoptosis](image)

B

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- pJAK2
- JAK2
- p-STAT5 (Tyr694)
- STAT5
- p-STAT3 α/β (Tyr705)
- STAT3
- p-AKT(Ser473)
- AKT
- β-actin

C

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- p-STAT5
- STAT5
- STAT3
- p-AKT
- AKT
- β-actin
Figure 5

A

HEL 92.1.7

% apoptosis

Ctrl 500 nM TG 1000 nM TG 10 nM PS 500 nM TG + PS + PS

p=0.02

p=0.01

B

HEL 92.1.7 cells

Cl

0 0.2 0.4 0.6 0.8 1.0

Fractional Effect

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C

HEL 92.1.7

PS, 24 hours

μM TG, 24 hours

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<td>GATA1</td>
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</table>
Figure 6

A

% non-viable cells

untreated 200 nM TG 500 nM TG 1000 nM TG 20 nM PS + PS + PS + PS

B

Primary MF-MPN

0 10 20 50 100 nM, PS, 24 hours

JAK2 p-STAT5 STAT5 p-STAT3 α/β STAT3 α/β Bcl-xL p-ERK1/2 ERK1/2 p-AKT AKT Ac K69 hsp90 β-actin

C

CD34+ Primary MF-MPN cells

- + - + 0.5 µM TG
- - + + 10 nM PS

p-STAT5 STAT5 p-STAT3 α/β STAT3 α/β β-actin

D

MF-MPN CD34+CD38-Lin n=3

% non-viable cells

Control 200 nM TG 1000 nM TG 20 nM PS 200 nM TG + PS 1000 nM TG + PS
Co-treatment with panobinostat and JAK2 inhibitor TG101209 attenuates JAK2V617F levels and signaling and exerts synergistic cytotoxic effects against human myeloproliferative neoplasm cells